Similarities in *Leptospira* Serogroup and Species Distribution in Animals and Humans in the Indian Ocean Island of Mayotte

Amélie Desvars,* Florence Naze, Gwenaël Vourc'h, Eric Cardinale, Mathieu Picardeau, Alain Michault, and Pascale Bourhy

Laboratoire de Bactériologie-Parasitologie-Virologie-Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint-Pierre, La Réunion, France; Unité Mixte de Recherche Contrôle des Maladies Animales Exotiques et Emergentes (UMR CMAEE), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Sainte-Clotilde, La Réunion, France; INRA, UR346 Unité d'Epidémiologie Animale, Saint-Genès Champanelle, France; Centre Régional de Recherche et de Veille sur les Maladies Emergentes de l'Océan Indien (CRVOI), Sainte-Clotilde, La Réunion, France; Institut Pasteur, Unité de Biologie des Spirochètes, Centre National de Référence de la Leptospirose, Paris, France

Abstract. Our objective was to identify local animal reservoirs of leptospirosis to explain the unusual features of *Leptospira* strains recently described among patients on the island of Mayotte. By means of a microscopic agglutination test using local clinical isolates, we found that 11.2% of black rats were seropositive to *Leptospira*, whereas 10.2% of flying foxes, 2% of lemurs, 93.1% of domestic dogs, and 87.5% of stray dogs were seropositive. As observed in humans, Mini was the main serogroup circulating in animals, whereas serogroup Icterohaemorrhagiae was absent. Using quantitative polymerase chain reaction, we also showed that 29.8% of rats carried leptospires in their kidneys. The sequencing of 16S rRNA gene sequences of *Leptospira* found in black rat kidneys identified four genomospecies (*Leptospira borgpetersenii*, *Leptospira interrogans*, *Leptospira kirschneri*, and *L. borgpetersenii* group B), which established black rats as the major source of leptospirosis transmission to humans. The origins of such a genetic diversity in *Leptospira* strains are discussed.

INTRODUCTION

Leptospirosis is a zoonotic disease that has a significant health impact worldwide, particularly in tropical and developing countries.[1] The disease can be life-threatening, with complications such as Weil's disease or severe pulmonary hemorrhage syndrome. Human infection results from exposure to infected urine of carrier mammals, either directly or by contaminated soil or water,[2] thus animal shedders pose a public health risk.

Leptospirosis is endemic in Mayotte. The annual incidence between 1984 and 1989 was 3.83 cases per 100,000 inhabitants but since 2007, diagnostic methods have been improved and annual incidence was reported to be higher than 20 cases per 100,000 inhabitants.[3] Pathogenic *Leptospira* strains responsible for clinical human cases showed a high genetic diversity. Moreover, serogroup Icterohaemorrhagiae has never been reported in humans and a new (probably endemic) strain called *Leptospira borgpetersenii* group B has been described,[3,4] which makes the epidemiology of leptospirosis in Mayotte unique. The reasons for the occurrence of this strain diversity remain to be uncovered. The main source of diversity originates probably in the animal reservoir hosts that infect humans.

Mayotte has a surface area of 376 km^2 and is a French overseas department. It is geographically part of the Comoros archipelago, located between northern Madagascar and northern Mozambique. Mayotte is characterized by a tropical climate that is hot, humid, and rainy during the monsoon season between November and May. The development of sanitary institutions is recent on the island, and self-subsistence agriculture and fishing are the principal sources of household income. The only native terrestrial mammals in Mayotte are Chiroptera (*Chaerephon pumilus*, *Taphozous mauritianus*, and the flying fox *Pteropus seychellensis*), thus the potential reservoir hosts are mostly introduced mammalian species: small mammals (the domestic mouse *Mus musculus*, the black rat *Rattus rattus*, the shrew *Suncus madagascariensis*, and the tenrec *Tenrec ecaudatus*), wild carnivores (the Indian civet *Viverricula indica* and stray dogs), primates (the brown lemur *Eulemur fulvus*), and domestic animal species (cats, dogs, zebus, and goats).

The aim of this study was to assess the potential of one endemic species (the flying fox) and three introduced species (the ship rat, domestic and stray dogs, and the brown lemur) to be reservoir hosts for leptospirosis in Mayotte. To achieve this objective, we studied first the seroprevalence of leptospirosis infection in these four commensal animal species by means of the microscopic agglutination test (MAT) using local clinical isolates.[3,4] These animal species were chosen because of their large population size and close contact with humans. Second, we focused on the main potential reservoir host—black rats—and compared the genetic diversity of the strains identified in this rodent species to those isolated from patients.[3,4] The origins of this unique genetic diversity among local *Leptospira* strains are discussed in this work. Molecular data provided new insights into the epidemiology of the disease on this tropical island.

MATERIALS AND METHODS

Field methods. Flying foxes were trapped in two sites by mist netting at nightfall, following previously described methods,[5] and 1 mL of blood was sampled from the humeral vein. Hemostasis at the venipuncture site was done by manual compression. Before release, flying foxes were given fruit juice to be fully hydrated.

Lemurs were anaesthetized using hypodermic syringes and a combination of tiletamine and zolazepam (Zolétil) at the recommended dosage of 8–10 mg·kg^{-1}.[6] The entry site of the hypodermic syringe and site of venipuncture were disinfected with povidone iodine. Ocular gel (Ocrygel) was put on the cornea to avoid dehydration. Body temperature, cardiac, and respiratory function of each lemur were monitored by veterinarians during anesthesia. If needed, post-induction

*Address correspondence to Amélie Desvars, Laboratoire de Bactériologie-Parasitologie-Virologie-Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France. E-mail: amel.desvars@gmail.com

supplementation was done by hand injection with Zolétil at 4–5 mg·kg^{-1}. Three to 3.5 mL of blood was sampled from the jugular vein and animals were released on the site of capture after complete recovery.

Domestic dogs were sampled by private local veterinarians at classical venipuncture sites after oral agreement with the owners. Stray dogs were caught by the Brigade Nature of Mayotte and sampled in the field.

Rats were trapped using baited-live traps (Manufrance) laid overnight. Rats were euthanized by injection of pentobarbital, following the recommended procedure.[7] For each rat, an intracardiac blood puncture was performed and the kidneys were aseptically removed.

All blood samples were centrifuged and sera were collected. Sera and kidneys were frozen at –80°C for conservation until analyses.

Serological analysis. Live leptospiral organisms were used for the MAT following standard procedure.[8] To link the epidemiology of animal leptospirosis to the human disease, we used nine strains that were locally isolated from infected patients from Mayotte between 2007 and 2010[3,4] (Table 1). Except for strain 200803703, which was isolated from an imported case from Madagascar, the other strains were autochthonous. The reference strain Copenhageni from serogroup Icterohaemorrhagiae was included in the panel, and strain Hond Utrecht IV from serogroup Canicola was also included in the panel for dog sera because serogroups Canicola and Icterohaemorrhagiae are the only two serogroups included in the French vaccine for dogs. All sera showing agglutination underwent further 2-fold dilutions in a range of 1:100–1:12,800. We set the cut-off point at 1:100 for positive sera. We considered the serogroup with the highest titer to be the presumptive infecting serogroup and serum with this result was classified as being infected with single *Leptospira* serogroup. However, if two or more serogroups induced the highest titer, then, either the infection was caused by multiple serogroups, or the multiple agglutinations resulted from cross-agglutinations (titers not shown).

Real-time quantitative polymerase chain reaction (PCR). DNA was extracted from 20 to 25 mg of kidney tissue using the Qiagen Dneasy Blood and Tissue Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

To control for DNA extraction and to detect the presence of PCR inhibitors in DNA extracts, we amplified a 250-pb fragment of the housekeeping gene *gapdh* using the hamster GAPDH C/D primer pair (Table 2) under standard conditions.[9]

Detection of *lipL32* was performed by a Taqman as previously described by Stoddard and others (Table 2).[10] Real-time PCR were performed using the LightCycler480 (Roche Diagnostics, Meylan, France).

Characterization of Isolates by Partial 16S rRNA gene sequencing. A partial sequence of the *rrs* gene from positive kidney tissue samples was amplified by nested PCR using Taq polymerase (GE Healthcare, Buckinghamshire, UK) and primers A/B, and then C/RS4 (Table 2)[11,12] under standard conditions. Sequencing was performed at the Platform Genotyping of Pathogens and Public Health (Institut Pasteur, Paris, France) and sequences were aligned in GenBank using the basic local alignment search tool (BLAST: http://www.ncbi.nlm.nih.gov/BLAST) or using ClustalW2.

Sampling authorization and ethics statement. The sampling was part of the ChikAni research program managed by the Institut National de Recherche Agronomique (INRA) in which all experimental procedures were approved by the Animal Health Division of INRA. Animal work in Mayotte was legally approved by the Prefect of Mayotte and the Direction of Agriculture and Forestry of Mayotte, by prefectural decrees no. 005/CAB/2007 and no. 029/DAF/sef/2007 for sampling on stray dogs and on wildlife, respectively. *Eulemur fulvus* is listed in Appendix I and *Pteropus seychellensis* is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Permits to import samples from these species in Reunion Island, and then to export them to France were obtained (permits to import no. FR0797400112-I and FR0797400111-I, delivered on April 16, 2007, and permits to export no. FR0797600003-E and FR0797600004-E, delivered on October 16, 2007, for lemurs and fruit bats, respectively). Rats are introduced as invasive mammals on Mayotte,[13] thus no particular authorization is required for their capture and study. Euthanasia of animals was conducted using ethical methods following the recommended procedures of the Parliament and the Council of the European Union.[7] Euthanasia of stray carnivores was conducted ethically by sworn agents of the Brigade Nature

TABLE 1
Species, serogroups, serovars, and reference strains of *Leptospira* used as antigens for the MAT in this study

Strain denomination*	Species	Serogroup	Possible serovar (or related serovar)[3,4]	Sequence type defined by MLST[3]
200701203	*L. borgpetersenii*	Mini	Beye/Mini	ST1
200901118	*L. borgpetersenii*	Pomona	Mozdok	ST10
201001127	*L. borgpetersenii* group B	Pyrogenes	Not determined	ST5
200901122	*L. borgpetersenii* group B	Pyrogenes	Kenya	ST5
201001125	*L. interrogans*	Pyrogenes	Not determined	ST2
200901489	*L. interrogans*	Pyrogenes	Undetermined (Pyrogenes/Camlo/Robinsoni)	ST2
Wijinberg†	*L. interrogans*	Icterohaemorrhagiae	Copenhageni	–
Hond Utrecht IV†	*L. interrogans*	Canicola	Canicola	–
200803703	*L. kirschneri*	Mini	Undetermined (Borincana/Unipertama/Hebdomadis)	ST9
201001128	*L. kirschneri*	Grippotyphosa	Not determined	ST13
200801774	*L. kirschneri*	Grippotyphosa	Grippotyphosa	ST6

*Denomination from Institut Pasteur, Paris.
†Reference strains.

TABLE 2
Primer and probe sequences used in this study

Primers or probe	Sequences	References
Hamster GAPDH C	5′-TGGTATCGTGGAAGGACTCA-3′	9
Hamster GAPDH D	5′-CCTGCTTCACCACCTTCTTG-3′	9
LipL32–45F	5′-AAGCATTACCGCTTGTGGTG-3′	10
LipL32–286R	5′-GAACTCCCATTTCAGCGATT-3′	10
LipL32–189P (probe)	FAM-5′-AAAGCCAGGACAAGCGCCG-3′-BHQ1	10
A	5′-GGCGGCGCGTCTTAAACATG-3′	11
B	5′-TTCCCCCCATTGAGCAAGATT-3′	11
C	5′-CAAGTCAAGCGGAGTAGCA-3′	11
RS4	5′-TCTTAACTGCTGCCTCCCGT-3′	12

of Mayotte and with respect of the French law no. 99-5 dated January 6, 1999 concerning *"dangerous and stray animals and protection of animals."*

RESULTS

All samples were collected between 29 March and 13 May 2007 during the warm and rainy season. A total of 292 animals (156 rats, 50 brown lemurs, 49 flying foxes, 29 domestic dogs, and 8 stray dogs) were sampled principally in rural areas close to human habitations (Figure 1). Blood was collected for all lemurs, bats, and dogs, but 31 rats could not be blood sampled. Kidney tissues were sampled in all 156 rats.

Five adult flying foxes (10.2%) were found seropositive for *Leptospira* antibodies. Four gave seropositivity for serogroup Pyrogenes (titers 200 to 400), whereas one was seropositive for serogroup Grippotyphosa at titer 200 (Table 3).

One adult male brown lemur (2%) was seropositive against two strains belonging to serogroup Pyrogenes (titers 200 and 400) (Table 3).

Twenty-two domestic dogs had up-to-date vaccination (< 1 year) against leptospirosis, one vaccinated dog was seronegative, and 10 animals had positive titers to more than one serogroup among which were Pomona and Grippotyphosa (Table 3). Ten vaccinated animals were seropositive to serogroup Icterohaemorrhagiae (titers 100 to 12,800) and one was seropositive to Canicola (titer 200). Vaccination could explain the high titers found against these two serogroups as they are included in the bivalent vaccine. Six out of seven (85.7%) non-vaccinated domestic dogs were seropositive (Table 3). Seropositive reactions were found against serogroups Icterohaemorrhagiae (1 of 6) at titer 800, Canicola (1 of 6) at titer 100, Mini (2 of 6) at titers 200 and 800. Seven out of eight stray dogs (87.5%) were seropositive against *Leptospira* (Table 3). Four stray dogs were seropositive to serogroup Mini (titers 200 to 6,400), one reacted to Pyrogenes (titer 400), and two were positive to Canicola (titer 100).

A total of 14 of 125 rats (11.2%) presented leptospiral antibodies, among which six presented coagglutinations (Table 3). The main serogroup circulating in the rat population in Mayotte was Mini (7 of 14 seropositive rats and titers comprised between 100 and 400). No serological reaction was found against serogroup Icterohaemorrhagiae. Antibodies against serogroup Grippotyphosa were recorded in three rats (titers 100 to 1,600), and one rat was seropositive to serogroup Pyrogenes (titer 200).

No DNA could be amplified from 15 kidney samples because of the presence of inhibitors (no amplification of the GAPDH control). Using real-time PCR, evidence of leptospiral infection was found in the kidneys of 42 out of 141 rats (29.8%). All Ct were found between 31.5 and 39.7 cycles.

Among the 42 LipL32-positive kidney samples, 20 were also PCR positive for *rrs* using nested PCR. Identification of the *Leptospira* species was achieved by sequencing of the 5′ variable region of the *rrs* gene and sequence analysis. The product sequence identified four pathogenic *Leptospira* species (Table 4): 9 of 20 samples were *L. borgpetersenii* (45%), 7 of 20 were *L. interrogans* (35%), 2 of 20 were *L. kirschneri* (10%), and 2 of 20 were *L. borgpetersenii* group B (10%), which was previously found in clinical isolates belonging to serogroup Mini. Alignment of leptospiral DNA from black rat kidneys and from humans showed perfect identity.

DISCUSSION

We determined the seroprevalence of leptospirosis infection for three introduced and one endemic mammal species to establish a link between human cases and potential animal reservoir hosts. None of the sampled animals presented any clinical signs of disease and positive titers reported in non-vaccinated wild species were generally low (≤ 400), which probably reflect earlier infections or continuous exposure to *Leptospira* environmental sources.

We reported a seroprevalence of 10.2% in fruit bats (*Pteropus seychellensis*) and identified Pyrogenes and Grippotyphosa as the infecting serogroups in this species. Smythe and others[14] found a higher seroprevalence of leptospirosis (28%) in pteropid bats from Australia and leptospiral DNA has been recovered from Australian fruit bat kidney and urine.[15] Direct contacts between fruit bats and humans are rare but *Pteropus* fruit bats can roost in village buildings[16] and may feed on cultivated plants including papaya, banana, guava, bread fruit, or mango[16] intended for human consumption. In addition, the grooming behavior of *Pteropus*, which consists in urinating and licking urine through the coat and wings,[16] favors direct contamination between bats. A recent study by Tulsiani and others[17] indicated the existence of a potential pathway for transmission of leptospires from pteropid fruit bats to rodents, by rodent contact with infected fruit bat urine.

This is the second report of the presence of anti-*Leptospira* antibodies in free-ranging non-human primates,[18] however the extent of direct *Leptospira* transmission between non-human primates and humans is unknown. In Barbados, the seroprevalence reported in apparently healthy wild vervet monkeys was 29.9%.[18] When non-human primates are naturally infected with *Leptospira*, either they develop a transient immunity and recover,[18,19] or they are hypersensitive and the
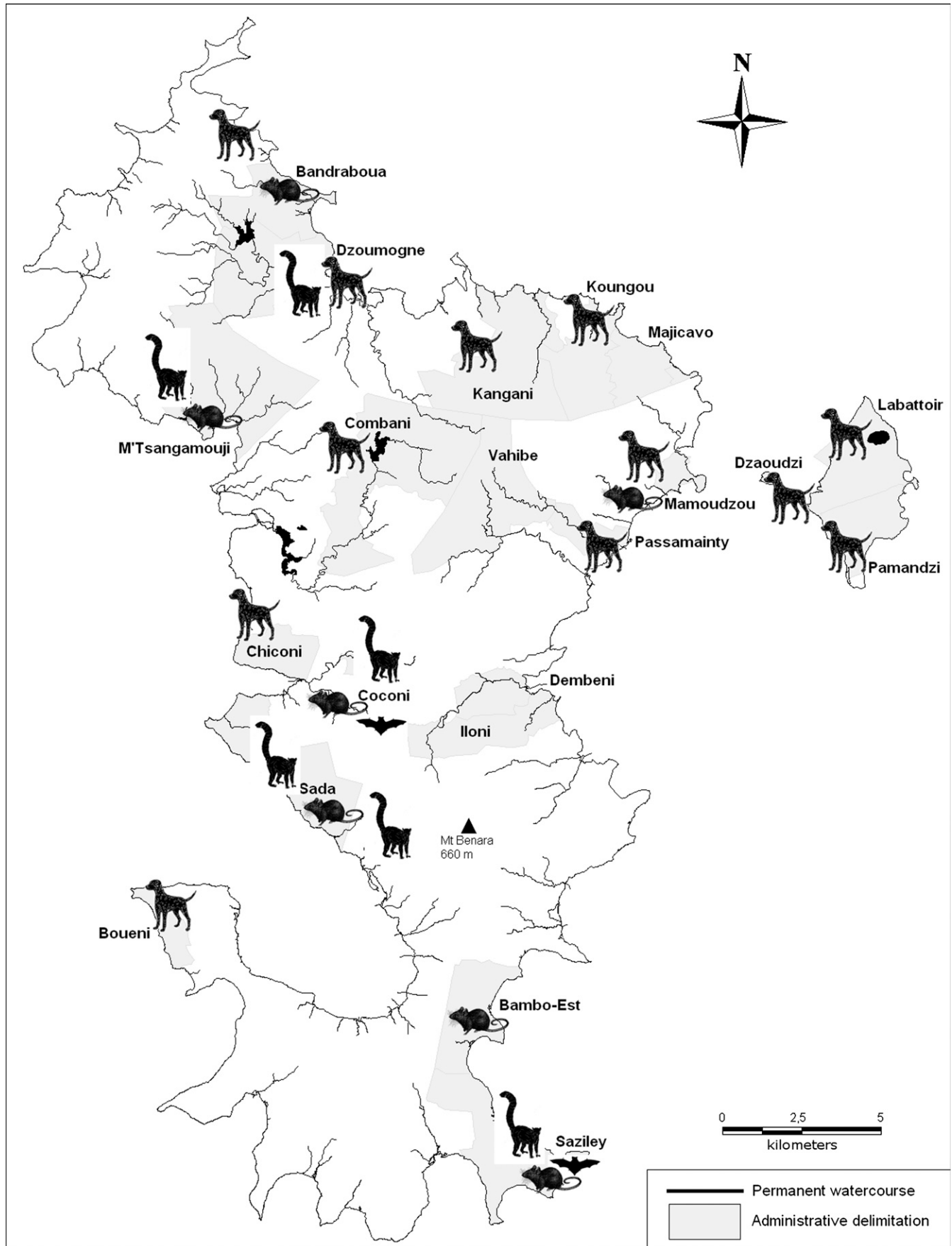
FIGURE 1. Cartography of Mayotte and sampling sites (S. Girard, ADEM-GESAM/CIRAD).

TABLE 3
Distribution of serogroup and seroprevalence by animal species (the minimum and maximum MAT titers are indicated in brackets)

	Black rats (*Rattus rattus*, N = 125)	Flying foxes (*Pteropus seychellensis*, N = 49)	Brown lemurs (*Eulemur fulvus*, N = 50)	Vaccinated* domestic dogs (N = 22)	Non-vaccinated domestic dogs (N = 7)	Non-vaccinated stray dogs (N = 8)
Mini†	7 (100–400)	–	–	–	2 (200, 800)	4 (200–6,400)
Pyrogenes‡	1 (200)	4 (200–400)	1 (200–400)	–	–	1 (400)
Grippotyphosa§	3 (100–1,600)	1 (200)	–	–	–	–
Icterohaemorrhagiae	–	–	–	10 (100–12,800)	1 (800)	–
Canicola	nd	nd	nd	1 (200)	1 (100)	2 (100)
Co-agglutinins¶	3	–	–	10	2	
Total seropositive (%)	14 (11.2%)	5 (10.2%)	1 (2%)	21 (95.5%)	6 (85.7%)	7 (85.7%)

*Up-to-date vaccination (< 1 year).
†Seropositive results and titers are considered together at the serogroup level for strains 200701203 and 200803703.
‡Seropositive results and titers are considered together at the serogroup level for strains 201001127, 200901122, 201001125, and 200901489.
§Seropositive results and titers considered together at the serogroup level for strains 201001128 and 200801774.
¶Animal seropositive for more than one serogroup at similar titers.
nd = not done.

disease is lethal (deadend hosts).[20–22] The low seroprevalence reported in lemurs (2%) suggests that infections are rare in this species, maybe because lemurs are almost completely arboreal and contacts with infected water are infrequent.

Our survey reported a seroprevalence of 93.1% in domestic dogs but this result is most likely biased because most of the domestic dogs had up-to-date (< 1 year) vaccination against leptospirosis (the bivalent vaccine available in France induces protection against serogroups Icterohaemorrhagiae and Canicola). We suggest that post-vaccinal titers persisted after annual (sometimes bi-annual) vaccinal injection and remain at high levels (> 1,600) because frequent exposures to field strains occur.[23–25] One "non-vaccinated" dog showed seropositivity at titer 800 to serogroup Icterohaemorrhagiae. This dog was an adopted adult, thus, we suggest that this animal may have previously been vaccinated. Stray dogs can prey on small mammals, which are recognized as the major reservoir host of leptospirosis,[8] however they can also be infected by the contaminated environment. We reported a seroprevalence of 85.7% in stray dogs showing that contact with *Leptospira* are frequent in this animal population and Mini was the main serogroup identified in stray and non-vaccinated domestic dogs. High antibody titer (6,400) to Mini serogroup in one stray dog suggests a possible recent infection. The number of stray dogs in Mayotte is very high (10,000 to 20,000 animals) and in consequence these animals may greatly contribute to *Leptospira* infection in humans.[26]

We showed a seroprevalence of 11.2% within the black rat population. We detected the presence of leptospiral DNA in 29.8% of rat kidneys, proving the role of the black rat in environmental contamination. Other studies reported a low genetic diversity among locally circulating *Leptospira* strains within the rat population.[27–32] Here, we showed that rats in Mayotte carried four genomospecies (Table 4) and that the genetic diversity among *Leptospira* strains infecting black rats is unique. *Rattus* sp. is considered as the preferred reservoir host for *L. interrogans* and *L. borgpetersenii*[28–30,32–34] and to our knowledge *L. kirschneri* (serovar Bim) has only been isolated from rats in Barbados.[35]

Our serological results corroborate serological observations and molecular data on human strains and confirmed that field strains from serogroup Icterohaemorrhagiae are absent on Mayotte.[3] Using MAT, Mini was found to be the major serogroup circulating in humans (70.2%), rats (50%), and non-vaccinated dogs (46.2%), which suggests that both these species could be a reservoir and transmission source of *Leptospira* to humans (Table 4). We also showed that the relative proportions of strains responsible for clinical cases in humans[3] and those carried in rat kidneys are similar (Table 4): *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* group B were found in 52.1%, 8.5%, 23.4%, and 15.9% of hospitalized patients and 45%, 35%, 10%, and 10% of rats, respectively. Moreover, we reported the perfect alignment between 16S rRNA partial sequences of *Leptospira* from rat kidneys and those isolated from infected patients. These data strongly suggest that the black rat population is the major reservoir and transmission source of *Leptospira* to humans on Mayotte. The relative percentage of the *L. interrogans* species was greater in black rats (35%) than in patients (8.5%) (Table 4). The study on human strains was

TABLE 4
Relative proportions of infecting serogroups in patients and wild non-vaccinated animals (MAT), and relative proportions of *Leptospira* species in patients and rats (16S rRNA sequencing of kidneys)

	Microscopic agglutination test (MAT) (No. positives by serogroup/total No. of positives)				16S rRNA sequencing (No. of strains identified by species/No. strains studied)			
Species (n = number studied)	Mini	Pyrogenes	Grippotyphosa	Pomona	*L. borgpetersenii*	*L. interrogans*	*L. kirschneri*	*L. borgpetersenii* group B
Lemurs (N = 50)	0/1 (0%)	1/1 (100%)	0/1 (0%)	0/1 (0%)	nd	nd	nd	nd
Pteropus bats (N = 49)	0/5 (0%)	4/5 (80%)	1/5 (20%)	0/5 (0%)	nd	nd	nd	nd
Non-vaccinated dogs (N = 15)	6/13 (46.2%)	1/13 (7.7%)	0/13 (0%)	0/13 (0%)	nd	nd	nd	nd
Rats*	7/14 (50%)	1/14 (7.1%)	3/14 (21.4%)	0/14 (0%)	9/20 (45%)	7/20 (35%)	2/20 (10%)	2/20 (10%)
Humans (N = 94)[3]	66/94 (70.2%)	17/94 (18.1%)	8/94 (8.5%)	3/94 (3.2%)	49/94 (52.1%)	8/94 (8.5%)	22/94 (23.4%)	15/94 (15.9%)

*MAT: 125 rats studied; 16S partial sequences: 20 rats.
nd = not done.

conducted on 94 isolates sampled over 3 years,[3] whereas rat sampling ($N = 20$) was conducted during a period of 6 weeks in 2007, which could explain the differences observed.

Animal populations of Mayotte have been introduced by settlers since the 8th century, first from East-Africa and then from Madagascar.[36,37] Tollenaere and others[38] showed that Mayotte presents (at least) three genetic variants of *R. rattus* that are very similar to Malagasy *R. rattus* genotypes but different from those of East Africa. Moreover, some *Leptospira* strains reported in Mayotte have previously been described in East Africa[3,39,40]: This may be indicative of a two-step colonization pattern: Mayotte was first colonized by the East African *R. rattus* carrying East-African leptospiral strains, but significant migrations from Madagascar occurred later. The Malagasy black rats could have driven out earlier East African occupants.[38] Consequently, *Leptospira* species first arrived with the East African *R. rattus*, and then may have infected introduced Malagasy black rats. The actual strains circulating in Mayotte might result from a genetic selection pressure, a selection by the hosts, and possibly DNA recombination events. The unique diversity of *Leptospira* on Mayotte could result from co-evolution between leptospires and its preferred reservoir host *R. rattus*. The presence of several genotypic variants of *R. rattus* could favor the specific adaptation of each *Leptospira* species to one particular *R. rattus* genotype.

We highlight the need to genetically characterize animal infecting strains to shed light on the global epidemiological risk for humans. The identification of 20 *Leptospira* sequences related to clinical isolates, the high density of black rats, and the poor sanitary conditions in much of Mayotte make the black rat the likely zoonotic source of leptospires. The black rat is a key element in the transmission cycle of leptospirosis and the reason for the maintenance of a remarkable and unique genetic diversity among *Leptospira* in Mayotte. Other animals are likely to play an important role in the transmission cycle of leptospirosis, in particular the zebus, which were not investigated in this study. Consequently, public health campaigns on preventive measures for the human population and management of rodents and stray dogs are crucial measures in the fight against leptospirosis.

Authors' addresses: Amélie Desvars, Laboratoire de Bactériologie-Parasitologie-Virologie-Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France; and Unité Mixte de Recherche Contrôle des Maladies Animales Exotiques et Emergentes (UMR CMAEE), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Sainte-Clotilde, La Réunion, France, E-mail: amel.desvars@gmail.com. Florence Naze and Alain Michault, Laboratoire de Bactériologie-Parasitologie-Virologie-Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France, E-mails: florence.naze@chr-reunion.fr and alain.michault@chr-reunion.fr. Gwenaël Vourc'h,

INRA, UR346 Unité d'Epidémiologie Animale, Saint-Genès Champanelle, France, E-mail: gwenael.vourch@clermont.inra.fr. Eric Cardinale, Unité Mixte de Recherche Contrôle des Maladies Animales Exotiques et Emergentes (UMR CMAEE), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Sainte-Clotilde, La Réunion, France, E-mail: eric.cardinale@cirad.fr. Mathieu Picardeau and Pascale Bourhy, Unité de Biologie des Spirochètes, Institut Pasteur, Paris, France, E-mails: mathieu.picardeau@pasteur.fr and pbourhy@pasteur.fr.

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